·Basic Research·

A comparison of three methods of decellularization of pig corneas to reduce immunogenicity

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Abstract

• AIM: To investigate whether decellularization using different techniques can reduce immunogenicity of the cornea, and to explore the decellularized cornea as a scaffold for cultured corneal endothelial cells (CECs). Transplantation of decellularized porcine corneas increases graft transparency and survival for longer periods compared with fresh grafts.

• METHODS: Six-month-old wild-type pig corneas were cut into 100-200 μm thickness, and then decellularized by three different methods: 1) 0.1% sodium dodecyl sulfate (SDS); 2) hypoxic nitrogen (N_2); and 3) hypertonic NaCl. Thickness and transparency were assessed visually. Fresh and decellularized corneas were stained with hematoxylin/eosin (H&E), and for the presence of galactose-α1,3-galactose (Gal) and N-glycolylneuraminic acid (NeuGc, a nonGal antigen). Also, a human IgM/IgG binding assay was performed. Cultured porcine CECs were seeded on the surface of the decellularized cornea and examined after H&E staining.

• RESULTS: All three methods of decellularization reduced the number of keratocytes in the stromal tissue by >80% while the collagen structure remained preserved. No remaining nuclei stained positive for Gal or NeuGc, and expression of these oligosaccharides on collagen was also greatly decreased compared to expression on fresh corneas. Human IgM/IgG binding to decellularized corneal tissue was considerably reduced compared to fresh corneal tissue. The cultured CECs formed a confluent monolayer on the surface of decellularized tissue.

• CONCLUSION: Though incomplete, the significant reduction in the cellular component of the decellularized cornea should be associated with a significantly reduced *in vivo* immune response compared to fresh corneas.

• **KEYWORDS:** cornea; decellularization; immune response; pig; xenotransplantation

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INTRODUCTION

A shortage of deceased human corneas for transplantation is a world-wide problem, especially in Asian countries. Prolongation of the average human's natural life-span and increasing refractory surgeries will aggravate the discrepancy between corneal demand and availability[1,2]. Penetrating keratoplasty (PKP) or partial thickness keratoplasty, such as Descemet's stripping endothelial keratoplasty (DSEK) and anterior lamellar keratoplasty (ALK), are the major treatment options for corneal blindness[3]. These procedures require fresh cadaveric tissue and therefore may involve waiting for weeks or months for a suitable graft to become available. In addressing this shortage, animal corneas, particularly pig corneas, are being investigated as an alternative source. The pig cornea has several similarities to the human cornea in anatomy and refractive properties [1,4]. In many cultures, pigs are acceptable as sources of organs, cells, and tissues[1].

However, there are hurdles to be overcome, such as the immune response to pig tissues and the biosafety of xenotransplantation[5,6]. The genetic distance between pig and human elicits an immune response that leads to rejection, even though the cornea is considered to be immune-privileged [7]. The first pig-to-nonhuman primate PKP using a fresh cornea was rejected within 15d, with extensive inflammatory cell infiltration and new vessel formation[8].

In vivo studies using decellularized pig corneas have demonstrated less rejection compared to fresh tissue [9-20] (Table 1). In the pig-to-nonhuman primate model, which is a more relevant model than studies in rodents or rabbits, two studies reported significantly prolonged graft survival compared to that of fresh corneas [19,20]. Regardless of the method of decellularization, better outcomes were reported with decellularized tissue. However, the reasons for the extended graft survival have not been elucidated. Shao *et al*[21]

Table 1 *In vivo* experiments using decellularized porcine cornea

Recipient	Decellularization method	Method	Control graft survival[1]	Decellularized graft survival	Reference
Rabbit	N_2 gas	ISPI		>6mo	[9]
Rabbit	0.1% SDS	ISPI		>12mo	[10]
Rabbit	Hypertonic NaCl	ALK	<4wk	>6mo	[11]
Rabbit	Lyophilization	ALK		Opacity cleared by 8wk and transparency maintained	[12]
Rabbit	0.5% SDS	ISPI		>24wk	[13]
Rabbit	Phospholipase A_2 and SD	ALK		>12mo	[14]
Rabbit	Lyophilization	ALK		Neovascularization and opacity cleared by 5wk, and transparency maintained	[15]
Rabbit	DNase, RNase	ISPI		>60d	[16]
Rabbit	0.5% SDS	ISPI		Opacity cleared within 8wk, and transparency maintained for >6mo	[17]
Rabbit	Ultrahigh-hydrostatic pressure	ISPI	<1wk	>8wk	[18]
Rhesus monkey	Dehydration ($CaCl_2$ silica gel)	ALK	15d	>6mo	[19]
Rhesus monkey	Hypertonic NaCl	ALK	<3-4wk (with steroid)	>6mo	[20]

ALK: Anterior lamellar keratoplasty; ISPI: Interlamellar stromal pocket implantation; [1]Control graft survival: Graft survival using the fresh cornea.

showed absence of galactose-α1,3-galactose (Gal) expression in decellularized corneal tissue, but to our knowledge no investigation of expression of the N-glycolylneuraminic acid (NeuGc) antigen or of human IgM or IgG binding has been reported.

Decellularized corneal tissue can be transplanted as a graft for ALK (*e.g.* to treat keratoconus or a corneal burn), but it can also serve as a scaffold for cultured corneal endothelial cells (CECs) as a DSEK graft to treat endothelial cell dysfunction (*e.g.* Fuchs' endothelial dystrophy). Decellularized tissue has the advantage of preserving the natural extracellular matrix, which is difficult to imitate using synthetic methods[22].

In this present study, we investigated whether decellularization can reduce the immunogenicity of pig corneas, thus leading to longer *in vivo* graft survivals that were shown previously [9-20]. We compared several different methods of decellularization, and also attempted to construct a DSEK graft composed of cultured pig CECs (pCECs) and decellularized cornea.

MATERIALS AND METHODS

Corneal Preparation Eyes of 6-month-old wild-type pigs were obtained from a local slaughterhouse, and the corneas were excised with at least 3 mm of scleral tissue. The corneal tissue was cut with a microkeratome (Moria, Doylestown, PA, USA) to a final thickness of 100-200 μm. In brief, the tissue was placed onto an artificial anterior chamber (Moria) and immobilized. The artificial anterior chamber was filled with balanced salt solution (Alcon, Mississauga, ON, Canada), and a tonometer (Ocular Instruments, Bellevue, WA, USA) was used to measure pressure, which for cutting is ideally 90 mm Hg. The epithelial layer was removed by rubbing the surface with a surgical swab (Moria) before cutting to reduce corneal thickness. The central corneal thickness was measured with a pachymeter (Tomey, Nagoya, Japan) and, depending on the corneal thickness, a suitable disposable blade (Moria) for the microkeratome was selected. After one cut, the thickness of the remaining tissue was measured again and the graft thickness was calculated. If the remaining tissue remained thicker than 100-200 μm, it was cut again. All animal tissues used in this study were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Decellularization of the Cornea The dissected pig corneas were randomly divided into 3 groups, with the following treatments (n=5 in each group): 1) Untreated fresh cornea (control); 2) The cornea was immersed in 0.1% sodium dodecyl sulfate (SDS; Sigma-Aldrich, St. Louis, MO, USA) at 37℃ for 7h [10]; 3) The cornea was placed in a 5 mL cryogenic storage vial (Fisher Scientific, Waltham, MA, USA) and N_2 from liquid nitrogen was poured into the vial to freeze the cornea and render the inside of the tube hypoxic; after fastening the cap tightly, the tube was maintained at room temperature for 7d [9]; 4) Corneas were immersed in 1.5 mol/L sodium chloride (NaCl, Sigma-Aldrich) at 37℃ for 24h, then placed in a solution of 0.05% trypsin (0.02% ethylenediaminetetraacetic acid, EDTA; Sigma-Aldrich) at 37℃ for 48h[11].

In two further groups, the N_2 method was further modified (n =3 in each group): 5) The cornea was irradiated (2800cGy) and then subjected to the same protocol as in (3) above; 6) The cornea was processed with N_2 and then treated with 4% Triton X-100 (Sigma-Aldrich) at 37℃ for 2h.

After each process, the tissues were washed with distilled water for 15min (with agitation) and then with phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA, USA) for 15min, repeated ×3.

Visual Assessment The thickness and clarity of the corneas

from each group were assessed visually. To investigate whether any opacity of the graft was reversible, the decellularized cornea was placed in 100% glycerol (Life Technologies, Carlsbad, CA, USA) for 2h and then reassessed.

Hematoxylin-eosin Staining The cornea was fixed with 10% formalin, stained with hematoxylin-eosin (H&E), and then examined by light microscopy (Nikon, Elgin, IL, USA).

Staining for Galactose-α1,3-galactose (Gal) and N-glycolylneuraminic Acid (NeuGc) Staining for Gal and NeuGc was performed as described previously[23]. Briefly, the cornea was embedded in optimal cutting temperature compound (Tissue-Tek, Miles Laboratories, Naperville, IL, USA) and stored at -80℃ until use. Frozen sections were cut to a 4 μm thickness, air dried for 1h, and fixed with acetone at -20℃ for 10min. The slides were washed with PBS followed by blocking with serum-free protein blocking solution (Dako, Carpinteria, CA, USA) for 10min at room temperature. Gal staining was carried out using fluorescein isothiocyanate (FITC)-conjugated BSI-B4 lectin (isolectin B4 from *Bandeiraea simplicifolia*, 10 mg/mL, Sigma-Aldrich) for 30min at room temperature and washed with PBS. NeuGc staining was carried out using a chicken-derived anti-NeuGc immunohistochemistry kit (Sialix, Cambridge, MA, USA), following the manufacturer's instructions. For nuclear staining, 4,6-diamidino-2-phenylindole (DAPI, Invitrogen) was applied. The stained tissues were examined by fluorescent microscopy (Nikon).

IgM and IgG Binding Assays IgM and IgG binding assays using human serum were carried out as described previously[23]. Sera were obtained from healthy human volunteers (n=5, including all ABO blood types) and pooled to form a single human serum reagent. The participants gave informed consent as per the guidelines of the Institutional Review Board of the University of Pittsburgh. Corneal samples were incubated with heat-inactivated, pooled human serum for 60min at room temperature. PBS was used for negative control samples. The slides were then washed with PBS and blocked with 10% goat serum for 30min at room temperature. FITC-conjugated goat-derived anti-human IgM (μ chain-specific) or IgG (γ chain-specific) polyclonal antibody (concentration 1:100; Invitrogen) for 30min at room temperature was used for the detection of IgM or IgG binding. DAPI was applied for nuclear staining and the slides were examined by fluorescent microscopy.

Seeding and Culture of Pig Corneal Endothelial Cells CECs from wild-type pigs were prepared and cultured as previously described [24]. Cells between passages 2 to 4 were used in all experiments. One hundred thousand (100 000) cells were seeded onto the surface of a decellularized cornea that was placed on the concave surface of a sterile cap of a 50 mL falcon tube (Fisher Scientific) with 100 μL of medium 199 (Invitrogen) containing 10% heat-inactivated FBS (Sigma-Aldrich), an antibiotic-antimycotic, and endothelial growth factor (30 μg/mL, BD Biosciences, San Jose, CA, USA). After 1d of incubation at 37℃, more medium was added and thereafter changed every 2 to 3d for 2wk. The cornea was washed gently with PBS and fixed with 10% formalin, then stained with H&E for examination.

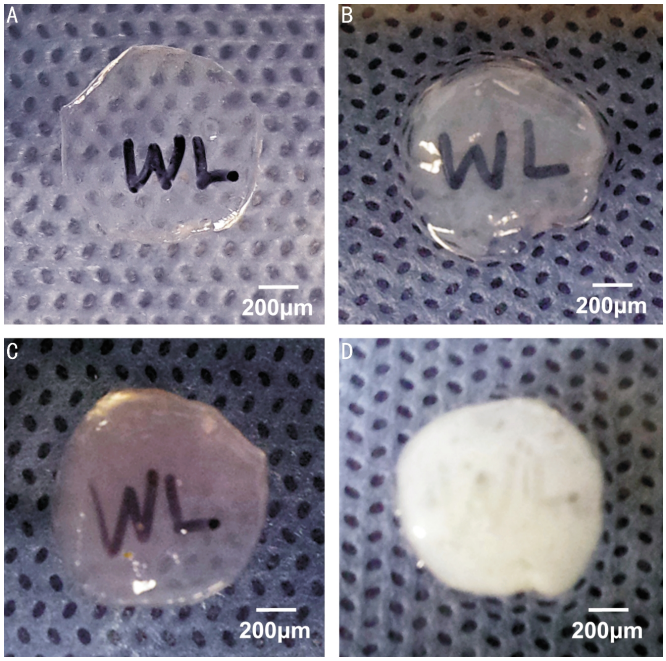

Figure 1 Visual assessment of fresh and decellularized pig corneas Macroscopic appearances of pig corneas before (A) and after decellularization with 0.1% SDS (B), N_2 (C), or hypertonic NaCl (D). Before decellularization, all tissues were cut with a microkeratome to a final thickness of 100-200 μm. Transparency was largely maintained in the corneas treated with SDS and N_2, while those treated with hypertonic NaCl lost their clarity due to severe edema, though transparency was restored by dehydration in glycerol for 2h.

RESULTS

Visual Assessment of Fresh and Decellularized Pig Corneas When visually assessed (Figure 1), pig corneas decellularized with 0.1% SDS (Figure 1B) or N_2 (Figure 1C) were transparent. They were edematous compared to fresh corneas (Figure 1A), but the edema was not as severe as those treated with hypertonic NaCl (Figure 1D), which lost its clarity and became severely swollen. Its clarity was restored by dehydration with glycerol for 2h (not shown). Additional treatment with irradiation or 4% Triton X-100 with N_2 did not improve or reduce the clarity compared to the N_2 method alone (not shown).

Hematoxylin-eosin Staining of Pig Corneas Before and After Decellularization Compared to fresh corneas (Figure 2A), treatment with 0.1% SDS (Figure 2B), N_2 (Figure 2C), or the combination of irradiation+N_2 (Figure 2E) resulted in a >80% reduction in keratocytes in the cornea. Treatment with 1.5M NaCl (Figure 2D) or the combination of N_2+Triton

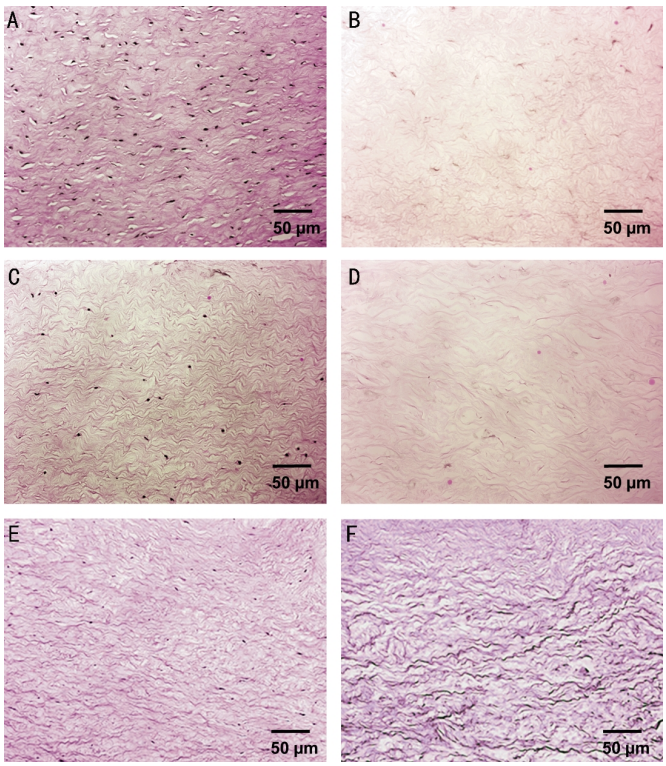

Figure 2 H&E staining of pig corneas before and after decellularization Representative images of H&E-stained pig corneas before (A) and after decellularization with 0.1% SDS (B), N_2 (C), hypertonic NaCl (D), combined N_2+irradiation (E), and combined N_2+Triton X-100 (F). In all cases, the number of keratocytes in the stromal tissue was reduced by >80% without distortion of the collagen structure. N_2 (C) did not efficiently remove the cellular components compared to 0.1% SDS or hypertonic NaCl, but its combination with irradiation (E) or Triton X-100 (F) resulted in a similar reduction.

X-100 (Figure 2F) removed the cellular components completely. The average numbers of keratocytes from 3 different microscopic fields (=0.08 mm^2) of each cornea were as follows: -fresh=208/field; SDS=18/field; N_2=28/field; N_2+irradiation=12/field, respectively. We could not detect remaining keratocytes in the hypertonic NaCl and Triton X-100 groups. Collagen structures were largely maintained and were not distorted in all groups.

Expression of Gal and NeuGc on Fresh and Decellularized Pig Corneas Fresh pig corneal stromal tissue had some Gal-positive keratocytes with weak positivity of the collagen structure (Figure 3A). No Gal-positive cells remained after decellularization (Figure 3B), even in the presence of some DAPI-stained nuclei. The expression of Gal was significantly decreased in the collagen tissue. The expression of NeuGc was also greatly reduced after decellularization. In the fresh porcine cornea (Figure 3C), strong expression of NeuGc was found in the stromal keratocytes as well as in collagen. However, after decellularization (Figure 3D) the remaining keratocytes were

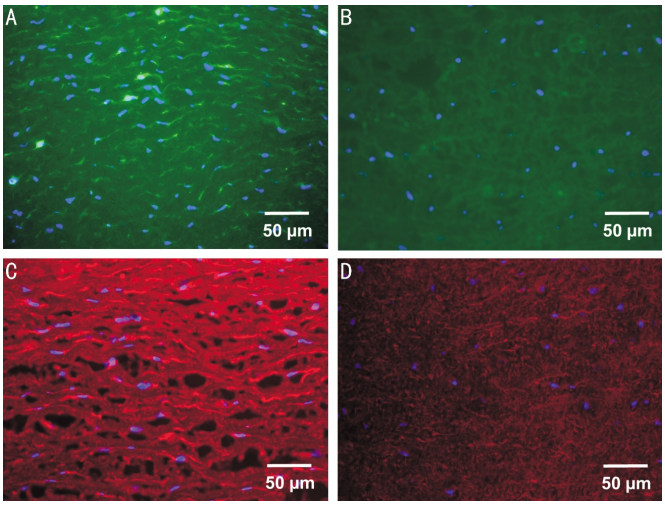

Figure 3 Gal and NeuGc immunostaining of fresh and decellularized pig corneas Gal (A, B) and NeuGc (C, D) staining of pig corneas before (A, C) and after (B, D) decellularization. Expression of Gal (BSI-B4 FITC: green) was significantly reduced after decellularization (B) compared to expression on fresh tissue (A). Expression of NeuGc (chicken anti-NeuGc antibody followed by biotinylated anti-chicken IgY and streptavidin Cy3: red) was also significantly reduced after decellularization (D) compared to expression on fresh tissue (C). DAPI (blue) was used for nuclear staining. The results are representative of at least 2 independent experiments. All methods of decellularization produced similar results.

negative for NeuGc, and expression in collagen was also decreased. There were no obvious differences between the decellularization methods tested (not shown).

Human IgM and IgG Antibody Binding of Pig Corneas Before and After Decellularization Binding of human anti-pig IgM to fresh cornea (Figure 4A) was decreased after decellularization (Figure 4B). In fresh tissue, binding was mainly to the keratocytes, which was not the case in the decellularized cornea. Binding of IgG was mainly to stromal collagen of fresh cornea, with a diffuse pattern (Figure 4C). This was greatly decreased in decellularized tissue (Figure 4D). There were again no obvious differences between the decellularization methods tested (not shown).

Cultured pCECs Form a Monolayer on the Surface of Decellularized Cornea To investigate whether the decellularized porcine cornea (Figure 5A) can provide a scaffold for cultured pCECs, pCECs were seeded on the surface of decellularized cornea and cultured for 2wk. Only the corneas treated with N_2 were used for this experiment (as N_2 yielded the best outcome with regards to removal of cells and persistent transparency). The pCECs formed a monolayer on the surface regardless of their original orientation (*i.e.* whether the cell surface had benn in contact with Descement's membrane or not. The absence of Descemet's membrane did not affect monolayer formation (Figure 5B).

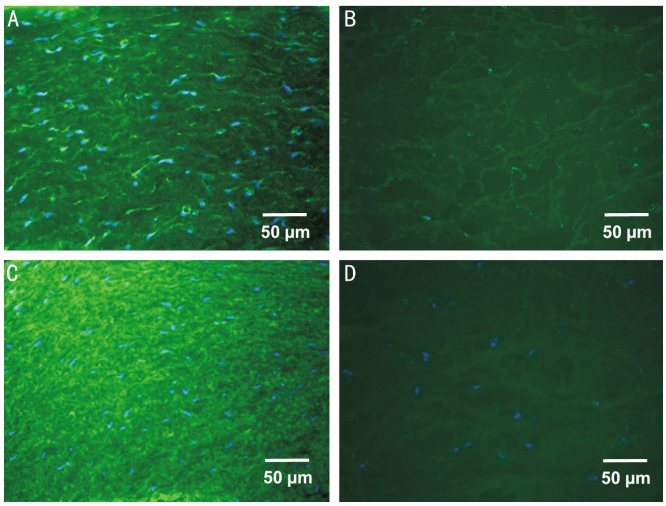

Figure 4 IgM and IgG binding assay of pig corneas before and after decellularization Human IgM and IgG antibody binding to pig corneas before (A, C) and after decellularization (B, D). Pooled human serum (20%) was incubated with the corneas, and antibody binding was detected by staining with FITC-conjugated anti-human IgM (A, B) or IgG (C, D) secondary antibody (green), with nuclear staining by DAPI (blue). Both IgM and IgG bound less to decellularized corneas (B, D) compared to fresh corneas (A, C). The results are representative of at least 2 independent experiments. All methods of decellularization produced similar results.

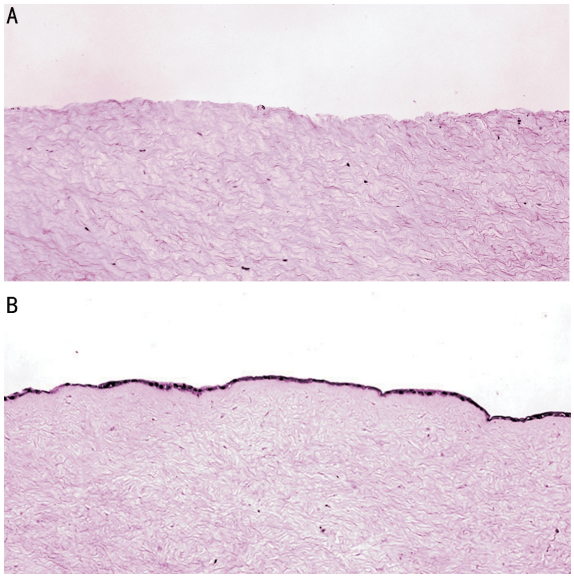

Figure 5 Cultured corneal endothelial cells form a monolayer on the surface of decellularized cornea Cultured pCECs formed a monolayer on the surface of N_2-decellularized corneas (from which the native CECs had been removed). The pCECs were seeded and cultured for 14d. Hematoxylin-eosin staining of decellularized cornea (A). The cultured CECs formed a confluent monolayer on the surface of denuded, decellularized corneal tissue (B).

DISCUSSION

Many methods have been proposed for effective tissue decellularization [25]. Owing to its special characteristics (*e.g.* transparency), decellularization methods for corneal tissue have to be modified from conventional methods [10,11,22]. The three techniques we tested have already been proven to be effective in removing the cellular components but preserving the collagen structure [9-11]. Moreover, in *in vivo* experiments in rabbits, decellularized grafts (using all three methods) demonstrated prolonged survival with less immunologic response[9-11].

Corneal stromal cells (keratocytes) are responsible for the well-organized transparent extracellular matrix of the cornea and are relatively quiescent in the adult cornea. However, once the cornea is injured, growth factors and cytokines, *e.g.* fibroblast growth factor 2 (FGF2), transforming growth factor-β (TGF-β), originating from corneal epithelial cells, inflammatory cells, and tear fluid, activate the keratocytes, which differentiate into fibroblasts and myofibroblasts (so-called 'activated keratocytes') [26]. This differentiation includes not only morphological change, but also *de novo* expression of extracellular matrix components that are not present in the normal cornea (*e.g.* type III collagen, tenascin-C, and matrix metalloproteinases). The changes also include downregulation or loss of expression of certain normal stromal components (*e.g.* keratan sulfate proteoglycans, prostaglandin D synthase).

Chen *et al* [27] investigated the Rho signaling pathway related to differentiation. Activated keratocytes seem to be responsible for wound healing and eventual loss of corneal clarity [28]. Hamrah [29] reported that the resident immature dendritic cells in the corneal epithelium and stroma lead to direct presentation of graft antigens to the host T cells and result in an immunologic response. These observations suggest that removal of the cellular components (*e.g.* keratocytes, dendritic cells) by decellularization may reduce graft immunogenicity and prolong graft survival.

Gal epitopes are known to be the major xenoantigens that cause hyperacute rejection in pig-to-primate xenotransplantation[30,31]. Recently, NeuGc, a non-Gal antigen that is widely expressed on the surface of cells (*e.g.* red blood cells, aortic endothelium) in all mammals except humans, has gained increased attention, and is considered an important xenoantigenic barrier [32,33]. Our previous report [23] and the present study indicate that the expression of NeuGc in the pig cornea is stronger than that of Gal. However, expression of both xenoantigens (Gal and NeuGc) was greatly reduced after decellularization. It is predictable that expression of any other (non-Gal, non-NeuGc) antigens, the nature of which remains unknown, might also have been reduced by decellularization, which might thus contribute to prolonged graft survival. IgM/IgG binding to the cornea was also significantly reduced after decellularization.

Pigs deficient in Gal and NeuGc have recently been reported [34]. Although the human humoral immune response to cells from these pigs was greatly reduced compared to that to wild-type pig cells, it was still significantly stronger than to

human allogeneic cells. Decellularization might provide immunologic advantages for tissue or cell xenotransplantation (*e.g.*, cornea, heart valve), even from genetically-engineered pigs. There are reports that decellularized pig heart valves, ligaments, and adipose tissues are less immunogenic than their fresh counterparts, both *in vitro* and *in vivo* [35-37]. Moreover, concerns regarding the potential for the transfer of an infectious microorganism will be diminished.

Endothelial keratoplasty, such as DSEK, is steadily replacing PKP in the USA as the treatment of choice for corneal endothelial dysfunction [38]. It has several advantages (*e.g.* minimizes the inflammation related to the presence of sutures, preserves corneal innervation, increases early visual recovery)[39]. However, the need for DSEK grafts far exceeds supply. There have been many attempts to culture CECs with or without carrier material for transplantation [40]. An ideal scaffold for cultured CECs should be non-cytotoxic, transparent, and have appropriate mechanical properties. Also it should allow cell repopulation on its surfaces, *e.g.* with CECs and stromal cells. Attempts have been made to repopulate the decellularized porcine cornea with human corneal cells, and all three cell types (epithelial, stromal, and endothelial) were successfully cultured and expanded[41]. The most critical cell type is the CEC, because these do not proliferate *in vivo* [42]. When we cultured pCECs on the surface of decellularized corneal tissue, they formed a monolayer as in a native cornea, suggesting that the decellularized cornea can provide a suitable scaffold for CECs. However, more investigation of CEC function (*e.g.* ZO-1 or Na^+K^+ ATPase staining) will be necessary. Since the major targets for the host cellular response are CECs, and injury of these cells is the leading cause of graft failure after corneal transplantation, *in vivo* experiments of DSEK in nonhuman primates will be necessary to evaluate grafts composed of decellularized stroma and cultured CECs.

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